BBA 65527

THE CRYSTALLIZATION AND PROPERTIES OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE ISOLATED FROM RABBIT MUSCLE BY A SIMPLIFIED PROCEDURE

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(Received July 18th, 1966)

SUMMARY

1. A simplified procedure for the crystallization of glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:DPN$^+$ oxidoreductase (phosphorylating), EC 1.2.1.12) from rabbit muscle is described.

2. The inactivation of this enzyme by reduced diphosphopyridine nucleotide has been studied with this preparation.

INTRODUCTION

This paper presents a rapid, reproducible method for the crystallization of glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:DPN$^+$ oxidoreductase (phosphorylating), EC 1.2.1.12)) of high specific activity from rabbit muscle. Such a method is desirable because experience with crystalline enzyme from a commercial source or that obtained by previous isolation methods has indicated great variability in the specific activity of the enzyme from one batch to another. Such variability poses difficulties because there is no convenient means to evaluate the effect of the inactive or extraneous protein. The procedure described in this paper involves a great saving in time and effort and has been conceived as a modification of the classical procedure[1]. As in a recent method[2], the enzyme isolated by this modification is of consistently higher specific activity and has increased stability on storage as compared with commercial preparations. Major points of difference are (1) the use of frozen rabbit muscle obtained locally, (2) utilization of a blender in extraction procedures, (3) precipitation with "enzyme grade" $(NH_4)_2SO_4$ to reduce the amount of heavy-metal ions added, (4) centrifugation *in lieu* of filtration, and (5) the use of complexing agents (EDTA and KCN) to reduce heavy-metal contamination and the addition of dithioerythritol to diminish the oxidation of sulfhydryl groups.

The present paper also represents a continuation of the work of previous publications[3-5] involved with the extensive inactivation of glyceraldehyde-3-phosphate dehydrogenase by reduced diphosphopyridine nucleotide (DPNH); studies

were carried out to evaluate the effect of pH and heavy metals on the inactivation process.

EXPERIMENTAL

Glyceraldehyde-3-phosphate (as the diethyl acetal), DPN^+ and DPNH were obtained from Sigma Chemical Co., dithioerythritol from Cyclo Chemical Corp., $(NH_4)_2SO_4$ (special enzyme grade) from Mann Research Laboratories, and Pel-Freeze frozen rabbit muscle from a local vendor. Enzymic activity was assayed by the method of KREBS[6] except that Tris–acetate buffer was substituted for pyrophosphate buffer. The crystalline enzyme as prepared exhibits 80% of total activity in the absence of cysteine and under these conditions, pyrophosphate was found to be inhibitory. The specific activity is defined in this paper as μmoles of DPN^+ reduced per min/mg protein. Absorbance readings in the assay were recorded on a Beckman DU spectrophotometer in the range where maximal linearity in the assay procedure is observed (approx. 0.2–0.5 min). In assaying for enzymic activity, an aliquot of the crystalline preparation was centrifuged at 24 500 $\times$ g for 20 min and the crystals were resuspended in deionized water to a final protein concentration of approx. 2.0 mg/ml. Protein determinations were carried out using a semi-micro biuret method[7].

RESULTS AND DISCUSSION

Purification of glyceraldehyde-3-phosphate dehydrogenase

Step 1. A total of 100 g of frozen rabbit muscle was broken into small pieces and added slowly into a Waring Blendor at 2° containing 250 ml of 0.03 M KOH–0.001 M EDTA. Once the muscle was dispersed, the final mixture was blenderized at high speed for a total of 4 min. The homogenate was centrifuged at 20 000 $\times$ g for 20 min; all subsequent centrifugations were carried out in like manner. The supernatant fluid (extract 1) was collected and the pellet was resuspended in 50 ml of 0.03 M KOH–0.001 M EDTA. The pellet was extracted by stirring for a few minutes and then centrifuged; the supernatant fluid (extract 2) was collected. Extracts 1 and 2 were combined and referred to as "crude extract".

Step 2. The "crude extract" was brought to 52% of saturation by the addition of a saturated solution of enzyme grade $(NH_4)_2SO_4$ (pH 7.5). The preparation was centrifuged and the supernatant fluid retained for Step 3.

Step 3. The supernatant fluid obtained in Step 2 was brought to 70% of saturation by the addition of solid enzyme grade $(NH_4)_2SO_4$ (the formula of KUNITZ[8] was used in all calculations of percentages of saturation). The preparation was allowed to stand 30 min at 0°. Following centrifugation, the supernatant fluid was retained for Step 4.

Step 4. The supernatant fluid was brought to 90% of saturation by the addition of solid $(NH_4)_2SO_4$ and allowed to stand 30 min at 0°. Following centrifugation, the precipitate was taken up in about 40 ml of EDTA, KCN and dithioerythritol, each being 10^{-3} M. The supernatant fluid contained no detectable activity.

Step 5. Two volumes of a saturated $(NH_4)_2SO_4$ solution, (pH 8.4) were added to the resuspended precipitate of Step 4. This solution was allowed to stand at 0°.

Crystals began to form within a few hours and crystallization was allowed to continue overnight.

Step 6. In recrystallizing the enzyme, the crystals were centrifuged and resuspended in about 20 ml of 10^{-3} M EDTA, KCN and dithioerythritol, after which 2 volumes of saturated $(NH_4)_2SO_4$ (pH 8.4) were added.

The purification procedure as summarized in Table I was repeated several times with only slight variation.

TABLE I

PURIFICATION SCHEME FOR THE CRYSTALLIZATION OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE FROM RABBIT MUSCLE

Fraction	*Volume (ml)*	*Units/ml*	*Total units*	*Protein (mg/ml)*	*Specific activity (units/mg)*	*Recovery (%)*
Crude extract	194	208	40 300	15.6	13.3	100
Supernatant fluid at 52% $(NH_4)_2SO_4$	380	105	39 700	4.8	21.4	98
Supernatant fluid at 70% $(NH_4)_2SO_4$	388	102	39 400	2.3	44.1	98
Resuspended precipitate of 90% $(NH_4)_2SO_4$ preparation	46	832	38 300	13.0	64.0	95
Crystalline preparation	124	234	29 000	2.8	83.5	72
1st recrystallized preparation	63	356	22 400	3.9	91.3	56
2nd recrystallized preparation	63	311	19 600	3.2	97.2	49
3rd recrystallized preparation	64	303	19 400	3.1	97.9	48

In the ultracentrifuge, the protein sedimented as a single symmetrical peak and gave an $s_{20,w}$ value of $7.92 \cdot 10^{-13}$ sec which agrees favorably with the recognized value for this enzyme[9]. The enzyme was found to be similar to all other preparations in regard to the amount of coenzyme bound as determined by its absorbance ratio, $A_{280m\mu}/A_{260m\mu}$, before and after treatment with charcoal[10,11].

DPNH-induced inactivation of the enzyme

Glyceraldehyde-3-phosphate dehydrogenase is inactivated when incubated with DPNH in air[3,4]. Since the present enzyme preparation was of higher specific activity and exhibited greater stability on storage in contrast to enzyme obtained commercially, it was subjected to inactivation by DPNH and in addition, the effect of heavy metals on the inactivation process was evaluated.

With the present enzyme preparation, inactivation induced by DPNH proceeds linearly with time and is essentially complete in 2 h. This finding differs from an earlier report[4] concerning studies conducted with a commercial enzyme preparation in which case there appeared to be an initiation step and the inactivation did not exceed 80% even after 4 h. Due to this apparent discrepancy, it does not appear proper to extrapolate these findings to a molecular level. However, it is probable that the commercial enzyme which routinely has a lower specific activity is a mixture of proteins whose interaction gives rise to this varied behavior.

The rate of inactivation is quite rapid when the pH of the incubation mixture is between 6 and 8.5. In this range, incubation mixtures buffered with arsenate,

phosphate or Tris–acetate all give similar results. At pH values below 6, DPNH is destroyed and therefore inactivation of the enzyme is less.

The inactivation, as shown previously[3–5], is accompanied by the loss of titratable sulfhydryl groups. The inactivation is prevented if the incubation mixture contains dithioerythritol or cysteine. If the enzyme is inactivated to a 50% level, the activity may be restored to about 75% by incubating the enzyme for 7 min with dithioerythritol or cysteine; however, if the enzyme is allowed to be inactivated extensively (2 h with DPNH), mercaptans will not restore the activity.

The inevitable question that has arisen before[5] and cannot be answered completely is whether or not heavy-metal ions mitigate the inactivation. It is known at this time that the addition of complexing agents (KCN, KSCN, EDTA, α,α'-dipyridyl, phenanthroline, and dithizone) has no effect on the inactivation induced by DPNH. Extraction of all solutions and rinsing of glassware with dithizone is ineffective in preventing the inactivation. Addition of heavy metals (Fe^{2+}, Fe^{3+}, Ni^{2+}, Mn^{2+}, Mg^{2+}, Cu^{2+}) has no effect in concentrations as high as 10^{-4} M. Cobaltous ion protects the enzyme against inactivation while Zn^{2+}, often implicated as an integral part of this enzyme, is without effect.

ACKNOWLEDGEMENTS

We would like to thank Mr. G. WEATHERBY for his assistance in conducting the ultracentrifugal studies and Dr. S. GRISOLIA for his suggestions. This investigation was supported by Public Health Service Grants No. A1 03505 and No. 5-T1-GM-492 and by Public Health Service General Research Support Grant 1 SO1 FR-05373. R.E.A. is a Research Career Development Awardee of the United States Public Health Service.

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